Direct Method for the Quantification of Free Saturated Fatty Acids in Tobacco

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A new approach to quantify free saturated fatty acids in tobacco using GC/MS/MS was investigated. Tobacco extracts were analyzed directly for free fatty acids without time-consuming clean up procedures and derivatization. Highly selective tandem mass spectrometric detection called selected reaction monitoring greatly improved the signal to noise ratio for underivatized saturated fatty acids and eliminated background interference. Therefore, the GC peaks of underivatized fatty acids were precisely integrated with improved peak shapes by using a polar stationary phase GC column. This method provides reliable and reproducible results with acceptable standard deviations.

Keywords: *Saturated fatty acids; gas chromatography; tandem mass spectrometry; selected reaction monitoring; tobacco*

INTRODUCTION

Saturated fatty acids exist in both plant and animal tissues esterified to glycerol as fats or lipids. The lipids comprise up to 7% of the dry weight of plant leaves (Gunstone, 1967), but free fatty acids are present in smaller quantities. Fatty acids are considered important ingredients or precursors of tobacco flavor. A wide range of saturated acids from volatile acetic acid to C23:0 long chain acid were found in tobacco leaves (Swain et al., 1962; Kimland et al., 1973; Lloyd et al., 1976). Analysis of fatty acids in tobacco smoke were also reported in the literature (Carruthers et al., 1959; Rao et al., 1988; Ha et al., 1991). Gas chromatography (GC) is the most used analytical technique for fatty acids analysis. There are several recent reviews describing gas chromatographic methods to quantitatively determine free fatty acids (Ackman, 1992; Larsson, 1994; Evershed, 1994). Most GC methods that quantify free fatty acids involve a number of preinjection sample treatments. The most common treatment is to derivatize carboxylic groups in free fatty acids to esters to make them more volatile. Sometimes, functional groups are introduced through the derivatization to improve the detection limits. Without derivatization, fatty acids eluting from a GC column show poorly resolved tailing peaks due to the interaction of the free acids with the stationary phase and column walls. Another important preinjection step for fatty acid analysis of a complex mixture is partial sample purification. Organic solvent extracts of dry plant leaves such as tobacco are very complex mixtures. Without sample clean up, derivatization may introduce some unwanted reactions and interfering artifacts. To quantify free fatty acids in complex mixtures without those preinjection treatments are considered not applicable due to the poor peak resolution and enormous chemical background. The process of sample clean up and derivatization makes the quantification process time consuming and tedious.

In this study, an approach to directly quantify free fatty acids in complex mixtures without doing sample clean up and derivatization is explored. In order to improve GC peak shapes, a polar polyethylene glycol bonded phase column (DB-WAX from J&W) is used. Free saturated fatty acids showed better peak shapes compared with a nonpolar polydimethylsiloxane column (DB-5 from J&W). The biggest obstacle in direct quantification of free fatty acids in complex mixtures is to overcome the interference of the chemical background from the crude extract. If the chemical background can be eliminated, a much better signal to noise ratio allows the quantification to be more accurate even when the GC peak has some tailing. Mass spectrometers with multiple analyzing devices, such as triple quadrupoles etc., provides great structure elucidation power and great selectivity when used in the tandem scanning mode (Gross, 1994; Biemann, 1993; Le Quere, 1993). It is possible to detect a specific compound or a group of specific compounds from a complex mixture using a selected reaction monitoring (SRM) scanning mode (Polettini et al., 1993; Okamoto et al., 1993; Buser and Mueller, 1994). By using the SRM scanning, saturated fatty acids in complex mixtures can be selectively detected. Therefore, chemical background is completely eliminated. Better shaped peaks with greatly improved sensitivity make it feasible to quantify underivatized fatty acids in complex mixtures.

EXPERIMENTAL PROCEDURES

Reagents and Tobacco Samples. Nine straight chain saturated fatty acids from decanoic acid to stearic acid were purchased from Aldrich Chemical Company (Milwaukee, WI). All standard acids had 99% or better purity and were used without further purification. GC-MS grade dichloromethane solvent was obtained from Fisher Chemical (Fair Lawn, NJ). Two dry tobacco samples of TR Madole 1994 crop year were obtained from the University of Tennessee Experimental Station in Springfield, TN. One tobacco sample was air-cured and the other was fire-cured. Both tobacco samples were ground without further drying, and a 2 mm screen was used to collect the ground tobacco.

Calibration Solutions. Except for undecanoic acid, a standard acid mixture was prepared by weighing 0.100 g of each of the eight saturated fatty acids from decanoic acid to stearic acid and dissolving them in a volumetric flask with 50 mL of dichloromethane. Undecanoic acid (0.100 g) was dissolved in 50 mL of dichloromethane in another volumetric flask and was used as the internal standard. Two sets of calibration solutions were prepared using these two standard solutions. The first set consisted of six solutions with concentrations of 2.0 μg/mL, 5.0 μg/mL, 10.0 μg/mL, 20.0 μg/mL, 40.0 μg/mL, and 60.0 μg/mL. Undecanoic acid solution was added to each one of them to a concentration of 20.0 μg/mL. The second set

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of calibration solutions consisted of five solutions with concentrations ranging from 0.20 μg/mL to 4.0 μg/mL. Undecanoic acid solution was added as the internal standard to a concentration of 2.0 μg/mL to each one of them.

Tobacco Extraction. Three sets of tobacco extracts were prepared. The first was prepared in the following way. Undecanoic acid (200 μg) was added to 1.00 g of ground fire-cured TR Madole tobacco in a capped vial. Fifty microliters of the standard fatty acids mixture (2.0 mg/mL each) was also added to the vial to the tobacco sample. Dichloromethane (10.0 mL) was added which made the concentration of spiked acids each 10.0 μg/mL. The vial was sealed and put in the ultrasonic bath and sonicated for 1 h at 35 °C. The dichloromethane extract was filtered with a nylon filter and dried over anhydrous sodium sulfate in preparation for GC/MS/MS analysis. Samples were prepared in duplicate. Two other tobacco extracts were prepared in the same way without spiking the tobacco sample with the standard fatty acids mixture.

The second set of tobacco extracts was prepared for both fire-cured and air-cured TR Madole tobacco. Undecanoic acid (100 μg) was added to the 1.00 g ground tobacco as the internal standard before the extraction. Dichloromethane (5.0 mL) was added, and the mixture was sonicated for 1 h. The internal standard, undecanoic acid, had a concentration of 20.0 μg/mL. The liquid phase was filtered very quickly to prevent possible solvent loss and was ready for direct GC/MS/MS analysis. Samples were prepared in duplicate.

The third sample set was made in a similar way as the second set. Five microliters of the 2.0 mg/mL undecanoic acid solution was added to 2.00 g of tobacco as the internal standard, and 10.0 mL of dichloromethane was added. The extraction time in the ultrasonic bath was 30 min. The liquid phase was removed, and another 5.0 mL of dichloromethane was added to the tobacco residue. The vial was sealed again and put back in the ultrasonic bath and sonicated 30 min. The liquid phase was removed, and the two extraction phases were combined. The combined extract was concentrated to a total volume of 5.0 mL and was ready for GC/MS injection. Samples were prepared in duplicate.

Instruments. A Hewlett Packard (Palo Alto, CA) 5890 GC was interfaced with a Finnigan MAT (San Jose, CA) TSQ 700 triple quadrupole mass spectrometer. The analytical GC column was 30 m long, 0.25 mm i.d., coated with 0.25 μm DB-WAX from J&W (Folsom, CA). The column was installed in the split/splitless injection port and operated in the splitless mode.

GC/MS/MS Analysis. The injection port temperature was 240 °C. The helium carrier gas flow rate was 1.15 mL/min. The temperature of the interface transfer line to the mass spectrometer was maintained at 250 °C. Initial oven temperature was 100 °C and was held for 1 min. The oven was then heated to 250 °C at a heating rate of 10 °C/min and was held at that temperature for 10 min. Injection volume was 2 μL.

Straight chain saturated fatty acids with a backbone of more than eight carbons generate a prominent ion at m/z 129^+ when ionized with electron impact (EI) at 70 eV. For branched saturated fatty acids, this ion also has a prominent intensity if the chain branched after C7. Acids investigated in this study were straight chain acids, and they all had intense m/z 129^+ peak in their EI mass spectra. Further fragmentation of this ion after collision with argon gas molecules generates a prominent ion, m/z 87^+, after losing a propylene group. Both m/z 129^+ and m/z 87^+ ions are fragments of fatty acids containing the carboxylic group. The SRM scanning mode was set in this way. GC eluents were ionized with EI energy of 70 eV. The m/z 129^+ ions were selected by the first quadrupole and collided with argon gas molecules in the second quadrupole. Fragment ions of the collision, m/z 87^+, were detected by the third quadrupole. With this scanning mode, only saturated fatty acids were shown in the chromatogram, and chemical background was eliminated. The argon gas pressure in the second quadrupole was 0.3 mtorr, and the collision energy was set at 25 eV. The ion source temperature was 150 °C, and the manifold temperature was 70 °C.

RESULTS AND DISCUSSION

Feasibility of the Direct GC/MS/MS Method for Saturated Fatty Acids. Quantitative measurement of fatty acids without derivatization have been reported only for short chain organic acids. There is no report on direct analysis of medium or long chain fatty acids in complex mixtures in the literature. Saturated fatty acids without derivatization show unacceptable GC peaks when a nonpolar capillary column is used. Specially treated bonded phase polar columns provide sharper and more symmetric peaks for free fatty acids (Sidisky et al., 1988). When a polar polyethylene glycol bonded phase column (DB-WAX from J&W) was used, free saturated fatty acids had better peak shapes compared with the nonpolar column (DB-5 from J&W). After using a polar phase DB-WAX column, there is an improvement in the peak resolution of the free acids.

For a triple quadrupole mass spectrometer, a precursor ion M1 from the compound of interest is selected by the first quadrupole and transmitted to the second quadrupole to collide with inert gas molecules, and a fragment ion M2 from the collision process is picked up by the third quadrupole. Only the compounds, which generate ion M1 in the ion source, collide with inert gas, and generate fragment ion M2, will be detected in this so-called SRM scanning mode. Therefore, if compounds generating M1 can be time resolved by way of GC, then the chemical background can be completely eliminated. Thus, the SRM scanning mode is highly selective. If the first analysis quadrupole is set to select the precursor ion m/z 129^+ from electron impact ionization and the third analysis quadrupole is set to detect the fragment, m/z 87^+, generated by collision in the second quadrupole, only saturated fatty acids with chain length longer than eight carbon atoms are detected. Figure 1 shows two GC/MS chromatograms of a dichloromethane extract of the fire-cured TR Madole tobacco. Chromatogram a is a total ion current trace with the mass spectrometer scanning from m/z 35^+ to m/z 500^+. This chromatogram showed a very complex mixture. Chromatogram b is a total ion current trace with SRM scanning for saturated fatty acids of the same extract. It is a clear and simple chromatogram only showing saturated fatty acid peaks. Palmitic acid is the most intense peak. Also seen in the chromatogram are fairly intense stearic acid and the internal standard, undecanoic acid. Myristic acid, pentadecanoic acid, and heptadecanoic acid also show prominent intensities. All free saturated fatty acids are well resolved. By comparing chromatogram b to a, it is obvious that the chemical background is completely eliminated when the SRM scanning mode is applied. Free fatty acids from the untreated tobacco extract show acceptable peak shapes with little tailing, and the excellent signal to noise ratio makes these peaks quantifiable.

For the first set of calibration solutions, the amounts of acids injected were 4.0, 10, 20, 40, 80, and 120 ng. Undecanoic acid was used as the internal standard because its natural abundance in tobacco is much smaller than other acids studied here. Table 1 shows the correlation coefficients of linear regressions for eight straight chain fatty acids. Calibration lines for decanoic acid, lauric acid, tridecanoic acid, and myristic acid have squared correlation coefficients of 0.9999. The squared correlation coefficients of the calibration lines for pentadecanoic acid, palmitic acid, heptadecanoic acid, and stearic acid are better than 0.99. These calibration lines

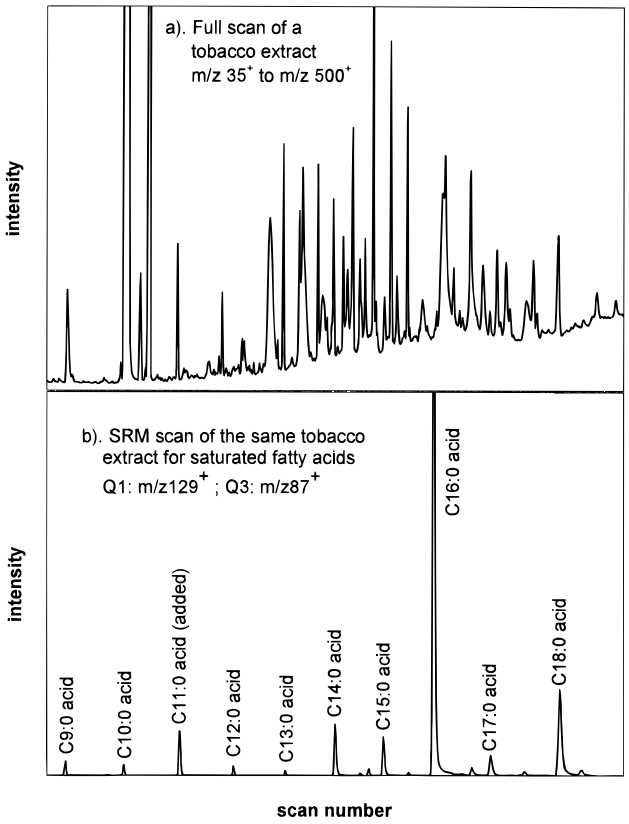

Figure 1. GC/MS chromatograms of a dichloromethane extract of fire-cured TR Madole tobacco: (a) full scan from *m/z* 35^+ to *m/z* 500^+; (b) SRM scan for saturated fatty acids of the same extract.

Table 1. Correlation Coefficients of Calibration Lines of Underivatized Saturated Fatty Acids (Undecanoic Acid Was Used as the Internal Standard)

	squared correlation coefficient (r^2)	
fatty acid	injection range 4.0−120 ng	injection range 0.4−8.0 ng
decanoic acid	0.9999	0.9998
lauric acid	0.9999	0.9988
tridecanoic acid	0.9999	0.9984
myristic acid	0.9999	0.9970
pentadecanoic acid	0.9998	0.9966
palmitic acid	0.9996	
heptadecanoic acid	0.9988	0.9823
stearic acid	0.9970	

were averaged between injections from 4.0 to 120 ng and from 120 to 4.0 ng.

The second set of calibration solutions were prepared in a lower concentration range. The amounts of each acid injected were 0.4, 1.0, 2.0, 4.0, and 8.0 ng. Squared correlation coefficients of calibration lines at this concentration range are also listed in Table 1. The squared correlation coefficients of calibration lines for decanoic acid, lauric acid, and tridecanoic acid are better than 0.99 in this concentration range.

The excellent correlation coefficients of the calibration lines indicates that direct injection of underivatized saturated fatty acids for quantitative purpose have an excellent linear response in the injection range from 0.4 to 120 ng. It is feasible to quantify fatty acids without derivatization or purification.

Quantification of Fatty Acids in Spiked Tobacco. The first set of tobacco extracts described in the Experimental Procedures was prepared to compare the concentration differences between the acids-spiked tobacco and unspiked tobacco. One gram of fire-cured tobacco was spiked with 100 μg for each of the eight straight chain fatty acids. Spiked concentration of the standard acids mixture was 10.0 μg/mL for each one of them after 10.0 mL of dichloromethane was added. Samples were prepared in duplicate for both spiked and unspiked samples. Average of six injections of two spiked extracts were compared with the average of six injections of two unspiked extracts. Table 2 lists the average concentrations for eight saturated fatty acids and their relative standard deviations for both spiked and unspiked samples. The relative standard deviation of the spiked extracts for decanoic acid, lauric acid, tridecanoic acid, and myristic acid are less than 5%. For pentadecanoic acid, palmitic acid, heptadecanoic acid, and stearic acid, the relative standard deviations are less than 10%. For the unspiked samples, six injections have the relative standard deviation for all the above mentioned acids less than 7% except C10:0, C12:0, and C13:0 acids, which have very low concentrations in the unspiked extracts. The repeated injection of both spiked and unspiked extracts show this GC/MS/MS method is quite reproducible since the relative standard deviations from six injections of two different samples are small.

The last column of Table 2 lists the concentration differences between the spiked samples and unspiked samples of the same fire-cured TR Madole tobacco. The concentration differences have a range from 10.2 μg/mL to 11.3 μg/mL for all the eight straight chain fatty acids listed. These values are very close to the spiked concentration, which is 10.0 μg/mL. This means that this GC/MS/MS method provides reliable quantitative results for free saturated fatty acids in complex tobacco extracts without any sample treatment.

Fatty Acid Content in Tobacco. The amount of C10:0−C18:0 straight chain saturated fatty acids in 1.00 g of tobacco was quantitatively determined and listed in Table 3. These data are averages of six injections from two different samples prepared in the same way.

Table 2. Fatty Acid Concentrations of Fire-Cured TR Madole Tobacco Spiked with Standard Fatty Acids Mixture and Unspiked Tobacco (Concentration Differences for All Listed Acids Are Close to 10 μg/mL, Which Is the Spiked Concentration)

	spiked sample		unspiked samples		concentration
fatty acid	concentration (μg/mL)	RSD, % (n = 6)	concentration (μg/mL)	RSD, % (n = 6)	difference (μg/mL) measured (spiked)
decanoic acid	11.38	2.6	0.21	25	11.2 (10.0)
lauric acid	11.65	2.5	0.31	30	11.3 (10.0)
tridecanoic acid	11.44	3.8	0.29	27	11.2 (10.0)
myristic acid	13.8	4.1	2.6	6.0	11.2 (10.0)
pentadecanoic acid	14.1	5.4	2.9	6.5	11.2 (10.0)
palmitic acid	51.7	5.2	41.5	6.1	10.2 (10.0)
heptadecanoic acid	14.2	8.2	3.6	6.6	10.6 (10.0)
stearic acid	24.1	10	13.4	6.9	10.7 (10.0)

Table 3. Free Saturated Fatty Acids in TR Madole Tobacco, Fire-Cured and Air-Cured

	TR Madole (air-cured)		TR Madole (fire-cured)	
fatty acid	concentration (ppm)	RSD, % ($n = 6$)	concentration (ppm)	RSD, % ($n = 6$)
decanoic acid	0.5	22	1.8	5.0
lauric acid	0.7	17	2.9	2.1
tridecanoic acid	0.2	12	2.0	5.0
myristic acid	17.4	2.5	19.2	6.7
pentadecanoic acid	22.0	2.7	23.0	4.8
palmitic acid	242.6	3.3	371.8	6.3
heptadecanoic acid	18.8	3.0	26.7	10.2
stearic acid	77.5	3.7	110.4	8.0

Table 4. Comparison of Saturated Fatty Acids in TR Madole Tobacco Extracted Once with Dichloromethane (1.0 g of Tobacco, 5.0 mL of Solvent) and Extracted Twice with Dichloromethane (2.0 g of Tobacco, 10.0 mL of Solvent and then 5.0 mL of Solvent) (Relative Standard Deviations Are Listed in Parentheses)

	air-cured TR Madole concentration (ppm)		fire-cured TR Madole concentration (ppm)	
fatty acid	1 g/5 mL extracted once	2 g/15 mL extracted twice	1 g/5 mL extracted once	2 g/15 mL extracted twice
myristic acid	17.4 (2.5%)	15.8 (3.9%)	19.2 (6.7%)	18.6 (3.0%)
pentadecanoic acid	22.0 (2.7%)	21.6 (5.4%)	23.0 (4.8%)	22.5 (3.5%)
palmitic acid	242.6 (3.3%)	238.6 (5.7%)	371.8 (6.3%)	391.4 (2.9%)
heptadecanoic acid	18.8 (3.0%)	19.7 (6.4%)	26.7 (10%)	25.3 (3.6%)
stearic acid	77.5 (3.7%)	82.6 (6.9%)	110.4 (8.0%)	109.2 (6.2%)

Palmitic acid is the most abundant saturated fatty acid in tobacco. In fire-cured tobacco, the average value for free palmitic acid is 372 ppm. The second most abundant acid is stearic acid which has an average of 110 ppm. Myristic acid, pentadecanoic acid, and heptadecanoic acid are at similar concentration levels in tobacco. Decanoic acid, lauric acid, and tridecanoic acid have relatively low concentrations. Their concentrations were calculated using the second set of calibration lines, which is more precise for small amount of acid injections (from 0.4 to 8.0 ng).

TR Madole is a variety of dark tobacco, and its free fatty acid contents has not been found in a literature search. In Swain and Stedman's study (1962), concentrations of free fatty acids in a fire-cured tobacco were 20 ppm for myristic acid, 320 ppm for palmitic acid, and 180 ppm for stearic acid. These values are close to the results of fire-cured TR Madole in this study. Semiquantitative measurements of fatty acids in a flue-cured British tobacco by Carruthers et al. (1959) showed that C15:0 acid was at the same level as C14:0 acid, but C17:0 acid had a significantly higher concentration than C14:0 and C15:0 acids.

In air-cured tobacco extracts, concentrations of C10:0, C12:0, and C13:0 acids are near the detection limit with large relative standard deviations. For fatty acids from myristic acid to stearic acid, the relative standard deviations are below 4%. Air-cured TR Madole tobacco contains lower concentrations of free saturated fatty acids than fire-cured tobacco of the same variety. Fire-cured TR Madole contains 40−50% more free palmitic acid, heptadecanoic acid, and stearic acid than the air-cured variety. This observation indicates that the fire curing process may increase the content of free saturated fatty acids in tobacco.

Free saturated fatty acids in tobacco were further evaluated by extracting the tobacco twice with dichloromethane. Two grams of tobacco was first extracted with 10.0 mL of dichloromethane and then with another 5.0 mL of dichloromethane. Two extracts were mixed and concentrated to a total volume of 5.0 mL. The amount of saturated fatty acids was measured and compared with results from a single extraction (1.0 g of tobacco extracted with 5.0 mL of dichloromethane). Results are listed in Table 4. The data were obtained from five injections of two samples prepared in the same way. The values in the parentheses are the relative standard deviations, which are all small for acids from C14:0 to C18:0. In Table 4, we can see that fatty acid concentrations from double extraction are very close to those from just single extraction. This means that a single extraction of 1.0 g of ground tobacco with 5.0 mL of dichloromethane is sufficient to quantitatively recover the free saturated fatty acids from the tobacco matrix. The comparison also confirms that the direct GC/MS/MS method for quantification of saturated fatty acids is reliable.

Conclusion. Saturated fatty acids in complex mixtures can be effectively quantified with GC/MS without derivatization or any other preinjection sample treatment. The highly selective SRM tandem mass spectrometric detection completely eliminates the chemical background of the mixture, and the signal to noise ratios are tremendously improved for free saturated fatty acids. Underivatized fatty acids have well resolved GC peaks when a polar DB-WAX column is used. Ultrasonic vibration used to extract saturated fatty acids from ground tobacco leaves is efficient and provides reproducible results.

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